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FILING DATE: *September 17, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/30806*

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US60/503,925*

UNITED STATES PATENT AND TRADEMARK OFFICE · DEPARTMENT OF COMMERCE

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 323 776 161 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Darcey	Clark	Woodinville, Washington 98072 USA
Kathleen	Keegan	Mercer Island, Washington 98040 USA
Scott	Peterson	Woodinville, Washington 98077 USA
Margaret	Weidner	Woodinville, Washington 98072 USA

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Methods for inhibition of aberrant cell proliferation using chk1 inhibitors

U.S.PTO 60/503925 091703

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

[x] Customer Number: 04743

OR

[ ] Firm or Individual Name	Greta E. Noland MARSHALL, GERSTEIN & BORUN LLP				
Address	233 S. Wacker Drive, Suite 6300 Sears Tower				
City	Chicago	State	IL	Zip	60606-6357
Country	US	Telephone	(312) 474-6300	Fax	(312) 474-0448

ENCLOSED APPLICATION PARTS (*check all that apply*)

[x] Specification *Number of Pages* 42
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[ ] Application Data Sheet. See 37 CFR 1.76
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[ ] Applicant claims small entity status. See 37 CFR 1.27.
[x] A check or money order is enclosed to cover the filing fees.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[x] No [ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

Date September 17, 2003

SIGNATURE Greta E. Noland

TYPED OR PRINTED NAME Greta E. Noland

REGISTRATION NO. *(if appropriate)* 35,302

TELEPHONE (312) 474-6647

Docket Number: 27866/39370

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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FEE TRANSMITTAL for FY 2003

Effective 01/01/2003. Patent fees are subject to annual revision.

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 160.00

Complete if Known	
Application Number	To be assigned
Filing Date	September 17, 2003 (herewith)
First Named Inventor	Darcey CLARK *et al.*
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Attorney Docket No.	27866/39370

METHOD OF PAYMENT *(check all that apply)*

☒ Check ☐ Credit Card ☐ Money Order ☐ Other ☐ None

☐ Deposit Account:

Deposit Account Number: 13-2855

Deposit Account Name: MARSHALL, GERSTEIN & BORUN LLP

The Director is authorized to: *(check all that apply)*

☐ Charge fee(s) indicated below ☒ Credit any overpayments

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☐ Charge fee(s) indicated below, **except for the filing fee** to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	750	2001	375	Utility filing fee	
1002	330	2002	165	Design filing fee	
1003	520	2003	260	Plant filing fee	
1004	750	2004	375	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	160.00

SUBTOTAL (1) ($) 160.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

			Extra Claims		Fee from below		Fee Paid
Total Claims		- =		x		=	
Independent Claims		- =		x		=	
Multiple Dependent						=	

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	84	2201	42	Independent claims in excess of 3
1203	280	2203	140	Multiple dependent claim, if not paid
1204	84	2204	42	** Reissue independent claims over original patent
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 0.00

***or number previously paid, if greater; For Reissues, see above*

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge – late filing fee or oath	
1052	50	2052	25	Surcharge – late provisional filing fee or cover sheet.	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	410	2252	205	Extension for reply within second month	
1253	930	2253	465	Extension for reply within third month	
1254	1,450	2254	725	Extension for reply within fourth month	
1255	1,970	2255	985	Extension for reply within fifth month	
1401	320	2401	160	Notice of Appeal	
1402	320	2402	160	Filing a brief in support of an appeal	
1403	280	2403	140	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive – unavoidable	
1453	1,300	2453	650	Petition to revive - unintentional	
1501	1,300	2501	650	Utility issue fee (or reissue)	
1502	470	2502	235	Design issue fee	
1503	630	2503	315	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	750	2809	375	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	750	2810	375	For each additional invention to be examined (37CFR 1.129(b))	
1801	750	2801	375	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	
				Other fee (specify)	

*Reduced by Basic Filing Fee Paid **SUBTOTAL (3)** ($) 0.00

SUBMITTED BY				(Complete *(if applicable)*)	
Name (Print/Type)	Greta E. Noland	*Registration No. (Attorney/Agent)*	35,302	*Telephone*	(312) 474-6647
Signature	Greta E. Noland			*Date*	September 17, 2003

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Dated: September 17, 2003 Signature: Richard Zimmermann (Richard Zimmermann)

Docket No.: 27866/39370

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT

Title:

METHODS FOR INHIBITION OF ABERRANT CELL PROLIFERATION USING CHK1 INHIBITORS

Darcey Clark
A U.S. Citizen

22904 61st Avenue S.E.
Woodinville, Washington 98072

Kathleen Keegan
A U.S. Citizen

5812 West Mercer Way
Mercer Island, Washington 98040

Scott Peterson
A U.S. Citizen

15003 225th Avenue N.E.
Woodinville, Washington 98077

Margaret Weidner
A U.S. Citizen

15726 160th Avenue N.E.
Woodinville, Washington 98072

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Dated: September 17, 2003 Signature: _____ (Richard Zimmermann)

METHODS FOR INHIBITION OF ABERRANT CELL PROLIFERATION USING CHK1 INHIBITORS

FIELD OF THE INVENTION

The present invention relates to improved methods for inhibiting aberrant cell proliferation involving the scheduling of administration of Chk1 activators (e.g., chemotherapeutic agents) and Chk1 inhibitors. At least one Chk1 activator is administered at a dose and for a time sufficient to induce substantial synchronization of cell cycle arrest in proliferating cells. Upon achieving substantial phase synchronization, at lease one Chk1 inhibitor is administered to abrogate the cell cycle arrest and induce therapeutic cell death. The invention is useful with any Chk1 activator and any Chk1 inhibitor, and finds application in treating or preventing cancerous and non-cancerous aberrant cell proliferation.

BACKGROUND

An important goal in healthcare is to develop and make available safer and more effective drugs and drug combinations for the treatment of aberrantly proliferating cells, such as for treatment of cancer. Most anti-proliferation therapies (including chemotherapy and radiation) act by disrupting vital processes such as DNA metabolism, DNA synthesis, DNA transcription, and microtubule spindle function, or by perturbing chromosomal structural integrity by introducing DNA lesions. These processes affect both normal and aberrantly proliferating (e.g., tumor) cells, however. As the maintenance of DNA integrity is essential to cell viability in normal cells, anticancer drugs have the lowest therapeutic index (i.e., the highest proportion of damage to normal cells as well as tumor cells) of any drug class.

Recent work has focused on ways to increase the therapeutic index of cancer and other anti-cell proliferation therapeutics. In this regard, cellular mechanisms, known as cell cycle checkpoints, have received attention. Individual cells create an exact copy of their chromosomes and then segregate each copy into two cells by a process called mitosis. Cells have sensing mechanisms, called cell cycle checkpoints, to maintain the order of these steps and to insure that each step is executed with high fidelity. [Hartwell *et al.*, *Science,* 246:629-634 (1989); Weinert *et al.*, *Genes and Devl,* 8:652 (1994).]

When cells detect DNA damage induced by a chemotherapeutic agent or by radiation, cell cycle checkpoints arrest the cell cycle, allowing time for the cells to repair the DNA damage, often to a point sufficient to continue proliferation and prevent cell death. For instance, the chemotherapeutic gemcitabine, a nucleoside analog, is incorporated into synthesizing DNA causing improper synthesis and inducing cell cycle arrest. If the cells could not overcome this cell cycle arrest, the cells would die. Some cancers appear to have generated a mechanism of overcoming this cell cycle arrest though. These resistant tumor cells simply accumulate in S phase while the chemotherapeutic agent is administered, and as soon as the drug is removed, repair the DNA damage and progress through the remainder of the cell cycle (Shi *et al., Cancer Res.* 61:1065-1072. 2001). The inhibition of DNA damage checkpoints is therefore expected to sensitize aberrantly proliferating cells to DNA damaging agents. Such sensitization is in turn hoped to increase the therapeutic index of such chemotherapeutic agents or radiation. Thus, Keegan *et al.*, (PCT/US02/06452, the contents of which are incorporated herein by reference), have disclosed certain small molecule compounds that selectively inhibit Chk1 kinase and their use in inhibiting Chk1.

The cell cycle is structurally and functionally conserved in its basic process and mode of regulation across all eukaryotic species. The mitotic (somatic) cell cycle consists of four phases, the G1 (gap) phase, the S (synthesis) phase, the G2 (gap) phase, and the M (mitosis) phase. The G1, S, and G2 phases are collectively referred to as interphase of the cell cycle. During the G1 phase, biosynthetic activities of the cell progress at a high rate. The S phase begins when DNA synthesis starts and ends when the DNA content of the nucleus of the cell has been replicated and two identical sets of chromosomes are formed. The cell then enters the G2 phase which continues until mitosis starts. In mitosis, the chromosomes pair and separate and two new nuclei form, and cytokinesis occurs in which the cell itself splits into two daughter cells each receiving one nucleus containing one of the two sets of chromosomes. Cytokinesis terminates the M phase and marks the beginning of interphase of the next cell cycle. The sequence in which the events in the cell cycle proceed is tightly regulated such that the initiation of one cell cycle event is dependent on the completion of the prior cell cycle event. This allows fidelity in the duplication and segregation of genetic material from one generation of somatic cells to the next.

It has been reported that cell cycle checkpoints comprise at least three distinct classes of polypeptides which act sequentially in response to cell cycle signals or defects in chromosomal mechanisms (Carr, A.M., Science, 271:314-315 (1996). The first class is a family of proteins which detect or sense DNA damage or abnormalities in the cell cycle. These sensors include Atm and Atr. The second class of polypeptides amplify and transmit the signal detected by the detector and is exemplified by Rad53 [Alen et al. Genes Dev. 8:2416-2488 (1994)] and Chk1. A third class of polypeptides includes cell cycle effectors such as p53 that mediate a cellular response, for example, arrest of mitosis and apoptosis.

Much of the current understanding of the function of cell cycle checkpoints has been derived from the study of tumor-derived cell lines. In many cases, tumor cells have lost key cell cycle check-points (Hartwell et al., Science 266: 1821-28, 1994). It has been reported that a key step in the evolution of cells to a neoplastic state is the acquisition of mutations that inactivate cell cycle checkpoint pathways, such as those involving p53 (Weinberg, R.A. Cell 81:323-330, 1995; Levine, A. J. Cell 88: 3234-331, 1997). Loss of these cell cycle checkpoints results in the replication of tumor cells despite DNA damage.

Noncancerous tissue, which has intact cell cycle checkpoints, typically is insulated from temporary disruption of a single checkpoint pathway. Tumor cells, however, have defects in pathways controlling cell cycle progression such that the perturbation of additional checkpoints renders them particularly sensitive to DNA damaging agents. For example, tumor cells that contain mutant p53 are defective both in the G1 DNA damage checkpoint and in the ability to maintain the G2 DNA damage checkpoint (Bunz et al., Science, 282:1497-501, 1998). Checkpoint inhibitors that target initiation of the G2 checkpoint or the S phase checkpoint are hoped to further cripple the ability of these tumor cells to repair DNA damage and, therefore, are candidates to enhance the therapeutic index of both radiation and systemic chemotherapy (Gesner, T., Abstract at SRI Conference: Protein Phosphorylation and Drug Discovery World Summit. March 2003.)

In the presence of DNA damage or any block to DNA replication, the checkpoint proteins Atm and Atr initiate a signal transduction pathway leading to cell cycle arrest. Atm has been shown to play a role in a DNA damage check-point in

response to ionizing radiation (IR). Atr is stimulated by agents that cause double strand DNA breaks, single strand DNA breaks, and agents that block DNA radiation.

Chk1 is a protein kinase that lies downstream from Atm and/or Atr in the DNA damage checkpoint signal transduction pathway. (Sanchez et al., Science, 277:1497-1501, 1997; U.S. Patent No. 6,218,109) In mammalian cells, Chk1 is phosphorylated in response to agents that cause DNA damage including ionizing radiation (IR), ultraviolet (UV) light, and hydroxyurea (Sanchez et al., supra; Lui et al., Genes Dev., 14:1448-1459, 2000). The phosphorylation and activation of Chk1 in mammalian cells is dependent on Atm (Chen et al., Oncogene, 18:249-256, 1999) and Atr (Lui et al., supra). Furthermore, Chk1 has been shown to phosphorylate both wee1 (O'Connell et al., EMBO J., 16:545-554, 1997) and Pds1 (Sanchez et al., Science, 286:1166-1171, 1999) gene products known to be important in cell cycle control.

These studies demonstrate that mammalian Chk1 plays a role in the Atm-dependent DNA damage checkpoint leading to arrest at S phase. A role for Chk1 in the S phase mammalian cells has recently been elucidated (Feijoo et al., J. Cell Biol., 154:913-923, 2001; Zhao et al., PNAS USA, 99:14795-800, 2002; Xiao et al., J Biol Chem., 278(24):21767-21773, 2003; Sorensen et al., Cancer Cell, 3(3):247-58, 2003) highlighting the role of Chk1 in monitoring the integrity of DNA synthesis. Chk1 invokes an S-phase arrest by phosphorylating Cdc25A, which regulates cyclinA/cdk2 (Xiao et al., supra and Sorensen et al., supra). Chk1 also invokes a G2 arrest by phosphorylating and inactivating Cdc25C, the dual specificity phosphatase that normally dephosphorylates cyclin-B/cdc2 (also known as Cdk1) as cells progress into mitosis (Fernery et al., Science, 277: 1495-7, 1997; Sanchez et al., supra; Matsuoka et al., Science. 282:1893-1897, 1998; and Blasina et al., Curr. Biol., 9:1-10, 1999). In both cases, regulation of Cdk activity induces a cell cycle arrest to prevent cells from entering mitosis in the presence of DNA damage or unreplicated DNA.

Additional classes of cell cycle checkpoint inhibitors inhibit the cell cycle at either the G1 or G2/M phase. UCN-01, or 7-hydroxystaurosporine, a derivative of staurosporine, was originally isolated as a non-specific kinase inhibitor, and was found to have its primary effect on protein kinase C, but has recently been found to inhibit the activity of Chk1 and abrogate the G2 cell cycle checkpoint (Shi et al., supra). Thus, UCN-01 is a non-selective Chk1 inhibitor. As a result, UCN-01 is

toxic to cells at high doses. At low doses, it non-specifically inhibits many cellular kinases and also inhibits the G1 checkpoint (Tenzer and Pruschy, Curr. Med Chem. Anti-Cancer Agents, 3:35-46, 2003).

UCN-01 has been used in conjunction with chemotherapeutic therapies, such as irradiation, the anti-cancer agent camptothecin (Tenzer and Pruschy, supra), and gemcitabine (Shi et al., supra) with limited success. In addition, UCN-01 has also been used to potentiate the effects of temozolomide (TMZ) induced DNA mismatch repair (MMR) in glioblastoma cells (Hirose et al., Cancer Res., 61:5843-5849, 2001). In the clinic, UCN-01 is not as effective a chemotherapeutic as once was hoped, perhaps due to a failure in treatment scheduling and a lack of identification of particular key molecular targets (Grant and Roberts, Drug Resistance Updates, 6:15-26, 2003). Thus, Mack et al. report cell cycle-dependent potentiation of cisplatin by UCN-01 in cultured non-small-cell lung carcinoma cell line, but do not identify with specificity the key cell cycle checkpoint(s) targeted by UCN-01. (Mack et al., Cancer Chemother Pharmacol., 51(4):337-348, 2003).

Scveral other strategies exist for sensitizing tumor cells to treatment with cell cycle affecting chemotherapeutics. For example, administration of 2-aminopurine abrogates multiple cell cycle checkpoint mechanisms, such as mimosine-induced G1 arrest or hydroxyurea-induced S phase arrest, allowing the cell to progress into and through mitosis (Andreassen et al., Proc Natl Acad Sci U S A., 86:2272-2276, 1992). Caffeine, a methylxanthine, has also been used to enhance cytotoxicity of DNA-damaging agents, such as cisplatin and ionizing radiation, by mediating progression through the G2 checkpoint and thereby inducing cell death. (Bracey et al., Clin Cancer Res., 3:1371-1381, 1997). However, the dose of caffeine used to accomplish the cell cycle abrogation exceeds clinically acceptable levels and is not a viable therapeutic option. Additionally, antisense nucleotides to Chk1 kinase have been used to increase sensitivity to the topoisomerase inhibitor BNP1350 (Yin et al., Biochem. Biophys. Res. Commun., 295:435-44, 2002), but demonstrate the problems typically associated with antisense treatment and gene therapy.

Thus, treatments that modulate the underlying molecular mechanisms of cell cycle progression and resistance to DNA damage were hoped to potentiate tumor cell killing and enhance the therapeutic index of existing therapies. Inhibition of additional DNA damage checkpoints by Chk1 inhibitors was hoped to potentiate

such treatments by selectively sensitizing abnormally proliferating cells to DNA damaging agents. However, the degree of selective sensitization or potentiation obtained was not as effective as hoped in these methods.

Consequently, there is a need in the art to develop a therapeutic regimen that more specifically targets particular cell cycle checkpoints in aberrantly dividing cells, thus providing better, faster and safer therapies to patients with proliferative diseases. The present invention addresses this need.

SUMMARY OF THE INVENTION

The present invention provides a method for inhibiting or preventing aberrant cell proliferation. The method comprises contacting a cell population comprising aberrantly proliferating cells with at least one Chk1 activator in an amount and for a time sufficient to substantially synchronize cell cycle arrest among the aberrantly proliferating cells. Upon achieving substantial synchronization of cell cycle arrest in said population, the cell population is contacted with at least one Chk1 inhibitor in an amount and for a time sufficient to substantially abrogate the cell cycle arrest.

In one embodiment, the present invention provides a method for sensitizing a population of aberrantly proliferating cells to the effects of at least one Chk1 activator. In another embodiment, the present invention provides a method for increasing the therapeutic index of at least one Chk1 activator in the treatment of at least one disease, condition, or disorder associated with, mediated by, or caused by aberrant cell proliferation.

The present invention also comprises articles of manufacture. Such articles comprise at least one Chk1 inhibitor, optionally together with a pharmaceutical carrier or diluent, and at least one label describing a method of use of the Chk1 inhibitor according to the invention. Such articles of manufacture may also optionally comprise at least one Chk1 activator.

The present invention also calls for use of a composition comprising at least one Chk1 inhibitor in the manufacture of a medicament for the inhibition or prevention of aberrant cell proliferation, or for the treatment or prophylaxis of a disease, condition, or disorder in a subject characterized or mediated by aberrant cell proliferation.

"Aberrant cell proliferation" means cell proliferation that deviates from the normal, proper, or expected course. For example, aberrant cell proliferation may include inappropriate proliferation of cells whose DNA or other cellular components have become damaged or defective. Aberrant cell proliferation may include cell proliferation whose characteristics are associated with a disease, condition, or disorder caused by, mediated by, or resulting in inappropriately high levels of cell division, inappropriately low levels of apoptosis, or both. Such diseases, conditions, or disorders may be characterized, for example, by single or multiple local abnormal proliferations of cells, groups of cells or tissue(s), whether cancerous or non-cancerous, benign or malignant, described more fully below.

"Inhibiting aberrant cell proliferation" means to slow or stop the rate at which aberrantly proliferating cells proliferate. This may result either from a decreased rate of replication, an increased rate of cell death, or both. Cell death may occur by any mechanism, including apoptosis and mitotic catastrophe. Use of the present invention may result in partial or complete regression of aberrantly proliferating cells, i.e., the partial or complete disappearance of such cells from the cell population. Thus, for example, when the population of aberrantly proliferating cells are tumor cells, the method of the invention may be used to slow the rate of tumor growth, decrease the size or number of tumors, or to induce partial or complete tumor regression.

"Preventing aberrant cell proliferation" means that the present invention may be used prophylactically to prevent or inhibit aberrant cell proliferation before it occurs, or to prevent or inhibit the recurrence thereof. Thus, in all embodiments, the invention may be used in vivo or ex vivo where no aberrant cell proliferation has been identified or where no aberrant cell proliferation is ongoing, but where aberrant cell proliferation is suspected or expected, respectively. Moreover, the invention may also be used in all its embodiments wherever aberrant cell proliferation has been previously treated to prevent or inhibit recurrence of the same. In these and related embodiments, the "cell population comprising aberrantly proliferating cells" may refer to any cell population where no aberrant cell proliferation has been identified or is ongoing, but where aberrant cell proliferation is suspected or expected, respectively, and/or any cell population previously treated for aberrant cell proliferation to prevent or inhibit recurrence of the same.

"Chk1 activator" means any known or after-discovered agent having the ability to activate Chk1 kinase activity in DNA repair and homeostasis at cell cycle checkpoints, and thus induce at least partial cell cycle arrest. Chk1 activators include those capable of arresting the cell cycle at any phase of the cell cycle, which phase may be referred to herein as the "target phase" for that activator. Target phases include any of the cell cycle phases except mitosis, that is the G1 phase, S phase, or G2 phase. A population of aberrantly proliferating cells may be contacted with one Chk1 activator or may be contacted with more than one Chk1 activator. If more than one Chk1 activator is used, the Chk1 activators may be co-administered or administered at separate times as determined by the attending physician or laboratory technician. Chk1 activators useful in the invention include DNA damaging agents, such as chemotherapeutic agents and/or radiation.

Any chemotherapeutic agent, known or after-discovered, capable of inducing substantial synchronization of cell cycle arrest in aberrantly proliferating cells may be suitable for use in the present invention and may be identified by means well known in the art. Examples of suitable chemotherapeutic agents include, but are not limited to: anti-metabolites such as 5-FU, gemcitabine, cytarabine, methotrexate, hydroxyurea, and 6-thioguanine; DNA-damaging agents; cytokines; covalent DNA-binding drugs such as platinum containing complexes; topoisomerase inhibitors such as camptothecin, irinotecan, topotecan, and etoposide; anti-tumor antibiotics such as the doxorubicin, actinomycin-C, daunorubicin, and bleomycin; differentiation agents; alkylating agents; methylating agents; nitrogen mustards; radiation sources optionally together with radiosensitizers and/or photosensitizers; or other commonly used therapeutic agents. In one aspect, the chemotherapeutic agent comprises gemcitabine, which typically induces S phase synchronization.

Additional examples of chemotherapeutic agents useful in the invention include hormones and antagonists thereof, radioisotopes, antibodies, natural products, and combinations thereof.

Non-limiting examples of these and other chemotherapeutic agents useful for the method of the present invention are listed in Table 1 below .

TABLE 1

Alkylating agents

Nitrogen mustards
mechlorethamine
cyclophosphamide
ifosfamide
melphalan
chlorambucil

Nitrosoureas
carmustine (BCNU)
lomustine (CCNU)
semustine (methyl-CCNU)

Ethylenimine/Methyl-melamine
thriethylenemelamine (TEM)
triethylene thiophosphoramide (thiotepa)
hexamethylmelamine (HMM, altretamine)

Alkyl sulfonates
busulfan

Triazines
dacarbazine (DTIC)

Antimetabolites
Folic Acid analogs
methotrexate
Trimetrexate
Pemetrexed
Multi-targeted antifolate

Pyrimidine analogs
5-fluorouracil
fluorodeoxyuridine
gemcitabine
cytosine arabinoside (AraC, cytarabine)
5-azacytidine
2,2'- difluorodeoxy-cytidine

Purine analogs
6-mercaptopurine
6-thioguanine
azathioprine
2'-deoxycoformycin (pentostatin)
erythrohydroxynonyl-adenine (EHNA)
fludarabine phosphate
2-chlorodeoxyadenosine (cladribine, 2-CdA)

Type I Topoisomerase Inhibitors
camptothecin
topotecan
irinotecan

Natural products

Antimitotic drugs
paclitaxel

Vinca alkaloids
vinblastine (VLB)
vincristine
vinorelbine

Taxotere® (docetaxel)
estramustine
estramustine phosphate

Epipodophylotoxins
etoposide
teniposide

Antibiotics
actimomycin D
daunomycin (rubido-mycin)
doxorubicin (adria-mycin)
mitoxantroneidarubicin
bleomycinsplicamycin (mithramycin)
mitomycinC
dactinomycin

Enzymes
L-asparaginase

Biological response modifiers
interferon-alpha
IL-2
G-CSF
GM-CSF

Differentiation Agents
retinoic acid derivatives

Radiosensitizers
metronidazole
misonidazole
desmethylmisonidazole
pimonidazole
etanidazole
nimorazole
RSU 1069
EO9
RB 6145
SR4233
nicotinamide
5-bromodeozyuridine
5-iododeoxyuridine
bromodeoxycytidine

Miscellaneous agents

Platinium coordination complexes
cisplatin
Carboplatin
oxaliplatin

Anthracenedione
mitoxantrone

Substituted urea
hydroxyurea

Methylhydrazine deriva-tives
N-methylhydrazine (MIH)
procarbazine

Adrenocortical suppres-sant
mitotane (*o,p'*- DDD)
ainoglutethimide

Cytokines
interferon (*, *, *)
interleukin-2

Hormones and antagonists

Adrenocorticosteroids/ antagonists
prednisone and equiv-alents
dexamethasone
ainoglutethimide

Progestins
hydroxyprogesterone caproate
medroxyprogesterone acetate
megestrol acetate

Estrogens
diethylstilbestrol
ethynyl estradiol/ equivalents

Antiestrogen
tamoxifen

Androgens
testosterone propionate
fluoxymesterone/equiv-alents

Antiandrogens
flutamide
gonadotropin-releasing hormone analogs
leuprolide

Nonsteroidal antiandrogens
flutamide

Photosensitizers
hematoporphyrin derivatives
Photofrin®
benzoporphyrin derivatives
Npe6
tin etioporphyrin (SnET2)
pheoboride-a
bacteriochlorophyll-a
naphthalocyanines
phthalocyanines
zinc phthalocyanines

Suitable Chk1 activators also include radiotherapeutic agents such as ionizing or ultraviolet radiation. Radiation Chk1 activators include, but are not limited to, gamma-radiation, X-ray radiation, ultraviolet light, visible light, infrared radiation, microwave radiation, and mixtures thereof. In one embodiment, such Chk1 activators may be administered in conjunction with at least one radiosensitizer and/or at least one photosensitizer.

"Chk1 inhibitor" means any compound or molecule, known or after-discovered whether naturally occurring or man-made, that is capable of at least partially abrogating cell cycle checkpoint activity of the Chk1 protein. Abrogation of cell cycle checkpoint is achieved when the cellular checkpoint mechanism(s) is (are) overcome sufficiently to allow the cell to pass from the cell cycle phase in which it is halted by the Chk1 activator to the next phase in the cell cycle or to allow the cell to pass directly to cell death. Abrogation of the cell cycle checkpoint permits cells to carry damage or imperfections, including damage induced by the Chk1 activator that might otherwise have been repaired, to subsequent cell cycle phases, thereby inducing or promoting cell death. Cell death may occur by any mechanism, including apoptosis and mitotic catastrophe. In one embodiment, Chk1 activators and Chk1 inhibitors that arrest and abrogate, respectively, the same cell cycle phase are used. Preferably, Chk1 inhibitors are selective Chk1 inhibitors, as described more fully below. A population of aberrantly proliferating cells may be contacted with one Chk1 inhibitor or may be contacted with more than one Chk1 inhibitor. If more than one Chk1 inhibitor is used, the Chk1 inhibitors may be co-administered or administered at separate times as determined by the attending physician or laboratory technician.

Chk1 inhibitors useful in the present invention include, but are not limited to, diarylurea compounds, e.g. aryl- and heteroaryl-substituted urea compounds described in co-owned, co-pending U.S. Patent Application No. 10/087,715; methylxanthines and related compounds (Fan et al., Cancer Res. 55:1649-54. 1995); ureidothiphenes (See International Patent Publ. No. WO03/029241); N-pyrrolopyridinyl carboxamides (See International Patent Publ. No. WO0/28724); antisense Chk1 nucleotides (see International Patent Publ. No. WO01/57206); Chk1 receptor antagonists (See International Patent Publ. No. WO00/16781); heteroaromatic carboxamide derivatives (See International Patent

Publ. No. WO03/037886); aminothiophenes (See International Patent Publ. No. WO03/029242); (indazolyl) benzimidazoles--(See International Patent Publ. No. WO03/004488); heterocyclic-hydroxyimino-fluorenes (See International Patent Publ. No. WO02/16326); scytoneman skeleton containing derivatives (scytonemin) (See U.S. Patent 6,495,586); heteroarylbenzamides (See International Patent Publ. No. WO01/53274); indazole compounds (See International Patent Publ. No. WO01/53268); indolacarbazoles (See Tenzer et al., supra); chromane deriviatives (See International Patent Publ. No. WO02/070515); paullones (see Schultz, et al., J. Med. Chem., Vol:2909-2919. 1999); indenopyrazoles (International Patent Publ. No WO99/17769); flavones (Sedlacek et al., Int J. Oncol. 9:1143-1168. 1996); peptide derivatives of peptide loop of serine threonine kinases (See International Patent Publ. No. WO98/53050): and oxindoles (See International Patent Publ. No. WO03/051838).

In one preferred embodiment, a selective Chk1 inhibitor is used in methods of the invention. "Selective Chk1 inhibitor" means a Chk1 inhibitor that will not function as a chemotherapy agent when administered alone. A non-selective Chk1 inhibitor, in contrast, may act as a chemotherapy agent by virtue of its ability to inhibit additional protein kinases or enzymes that are required for cell growth. This may result in additional cellular effects that lead to side effects and/or a reduced therapeutic index. Chk1 inhibitors of the present invention are therefore preferably selective Chk1 inhibitors.

Preferred Chk1 inhibitors as set out above preferably demonstrate at least 20 fold-selectivity in inhibiting Chk1 over the following protein kinases: protein kinase A, protein kinase C, cdc2 and pp60v-src. More preferred Chk1 inhibitors as set out above preferably exhibit at least 75 fold selectivity in inhibiting Chk1 over the following protein kinases: protein kinase A, protein kinase C, cdc2 and pp60v-src. Most preferred Chk1 inhibitors set out above preferably demonstrate at least 75 fold selectivity against protein kinase A, protein kinase C, cdc2, pp60v-src and protein kinase B/Akt-1, p38MapK, ERK1, p70S6K, cdc2, cdk2, chk2 and the abl tyrosine kinase. Fold selectivity is defined as the IC50 (see below) of the Chk1 inhibitor for the comparison kinase divided by the IC50 of the Chk1 inhibitor for Chk1. In one embodiment, the Chk1 inhibitor is not UCN-01.

Chk1 inhibitors useful in the invention can be used to enhance treatment of tumors that are customarily treated with an antimetabolite, e.g.,

methotrexate, 5-fluorouracil (5-FU) or gemcitabine. The method of the present invention comprises administration of a Chk1 inhibitor in combination with a Chk1 activator that can cause single- or double-strand DNA breaks or that can block DNA replication or cell proliferation. It is contemplated by the method of the invention that a therapeutically effective dose of the chemotherapeutic agent gemcitabine is in the range of about 0.1 mg/ m2 to about 1000 mg/m2 per dose. In one variation, gemcitabine is infused over time, ranging from about 0 to about 18 hours, at a concentration of about 10 mg/m2/min.

Compounds and pharmaceutical compositions suitable for use in the present invention include those wherein the active ingredient (e.g., Chk1 activator or Chk1 inhibitor) is administered in an amount effective to achieve its intended purpose. As used herein, a "therapeutically effective amount" or "dose effective to inhibit" means an amount effective to inhibit development of, or to alleviate the existing symptoms of, the subject being treated. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, which is expressed as the ratio of LD50 to ED50. Compounds that exhibit high therapeutic indices (i.e., a toxic dose that is substantially higher than the effective dose) are preferred.

Inhibition of the checkpoint kinase typically is measured using a dose-response assay in which a sensitive assay system is contacted with a compound of interest over a range of concentrations, including concentrations at which no or minimal effect is observed, through higher concentrations at which partial effect is observed, to saturating concentrations at which a maximum effect is observed. Theoretically, such assays of the dose-response effect of inhibitor compounds can be described as a sigmoidal curve expressing a degree of inhibition as a function of concentration. The curve also theoretically passes through a point at which the concentration is sufficient to reduce activity of the checkpoint enzyme to a level that is 50% that of the difference between minimal and maximal enzyme activity in the assay. This concentration is defined as the Inhibitory Concentration (50%) or IC50

value. Determination of IC50 values preferably is made using conventional biochemical (acellular) assay techniques or cell-based assay techniques.

Comparisons of the efficacy of inhibitors often are provided with reference to comparative IC50 values, wherein a higher IC50 indicates that the test compound is less potent, and a lower IC50 indicates that the compound is more potent, than a reference compound. Compounds useful in the method of the present invention demonstrate an IC50 value of about 20 nM or less when measured using the dose-response assay. In one aspect Chk1 inhibitor compounds demonstrate an IC50 value of less than about 10 μM. In another aspect Chk1 inhibitor compounds demonstrate an IC50 value of less than about 500 nM. It is further contemplated that Chk1 inhibitor compounds of the present invention demonstrate an IC50 value of less than about 250 nM, less than about 100 nM, or less than about 50 nM or less than about 20 nM.

The data obtained can be used in formulating a dosage range for use in humans. The dosage of such compounds preferably lies within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed, and the route of administration utilized.

The exact formulation, route of administration, and dosage is chosen by the individual physician in view of the patient's condition. Dosage amount and interval can be adjusted individually to provide plasma levels of the active compound that are sufficient to maintain desired therapeutic effects. In general, however, doses employed for adult human treatment typically are in the range of 0.001 mg/kg to about 100 mg/kg per day, in a range of about 0.1 mg/kg to about 500 mg/kg per dose.

The present invention may be applied to cell populations *in vivo* or *ex vivo*. *"In vivo"* means within a living subject, as within an animal or human. In this context, the invention may be used therapeutically in a subject to slow or stop the proliferation of aberrantly replicating cells. The invention may also be used as a prophylactic to prevent the occurrence or recurrence of aberrant cell proliferation or the manifestation of symptoms associated therewith. Other *in vivo* uses for which the invention may be therapeutic or preventative are described herein, or will become apparent to those skilled in the art.

"*Ex vivo*" means outside a living subject. Examples of *ex vivo* cell populations include *in vitro* cell cultures and biological samples such as fluid or tissue samples from humans or animals. Such samples may be obtained by methods well known in the art. Exemplary biological fluid samples include blood, cerebrospinal fluid, urine, saliva. Exemplary tissue samples include tumors and biopsies thereof. In this context, the invention may be used for a variety of purposes, including therapeutic and experimental. For example, the invention may be used *ex vivo* to determine the optimal schedule and/or dosing of administration of a Chk1 activator and Chk1 inhibitor for a given indication, cell type, patient, and other parameter. Information gleaned from such use may be used for experimental purposes or in the clinic to set protocol for *in vivo* treatment. Other *ex vivo* uses for which the invention may be suited are described below or will become apparent to those skilled in the art.

Chk1 activators useful in the invention increase the percentage of cells in their target phase of the somatic cell cycle, defined below. By way of background, cells in the somatic cell cycle typically cycle asynchronously. They are a dynamic population comprising cells in various phases of the cell cycle. The percentage of cells at any given phase in the cell cycle depends upon various factors, including, for example, cell type, environment, and cycle rate. Chk1 activator shifts these proportions, increasing the percentage of cells in the target phase for the activator, and decreasing the percentage of cells in the other phases. This shift in percentage may be referred to herein as synchronization, arrest, or piling up in the target phase.

The "target phase" of the cell cycle means the phase at which a Chk1 activator will cause a percentage of cells to increase. Different Chk1 activators may have different target phases. For example, ionizing radiation will increase the percentage of cells at the G2 phase. Thus, the G2 phase may be referred to herein as the target phase for ionizing radiation. The chemotherapeutic agents taxol and nocodazole will each increase the percentage of cells at the M phase. Thus, the M phase may be referred to as the target phase for taxol or nocodazole. Gemcitabine and low levels of camptothecin will each increase the percentage of cells at the S phase. Thus, the S phase may be referred to as the target phase for each of these chemotherapeutic agents. Any Chk1 activator having any target phase may be used in the present invention.

The proportion of cells in different phases of the cell cycle can be measured by those skilled in the art using any one of a variety of techniques. For example, a fluorescent DNA-binding dye, propidium iodide, can be used to distinguish cells in different cell cycle phases. Since cells in G2 have twice as much DNA as cells in G1, and S phase cells show an intermediate amount of DNA, the technique allows one to identify cells in different phases based on the DNA content of a cell. This method can be carried out on cell lines and tumor specimens (Cerra *et al.*, *Methods in Cell Biology, 33*:1-12, 1990) Furthermore, cells in S phase can be labeled with the nucleotide analog, bromo-deoxyuridine (BrdU) and then fixed and stained with an fluorescent-tagged antibody to BrdU. Both of these methods employ FACS to quantify the proportion of cells staining with these fluorescent markers.

An additional method for identification of cells in different phases of the cell cycle includes staining the cells with antibodies to markers that are either specific or selective for cell cycle phases. An antibody to the phosphorylated serine 10 residue of histone H3 is highly selective for mitotic cells. An antibody to phosphorylated serine 795 of the retinoblastoma protein, Rb, is selective for S phase cells (Connell-Crowley *et al., Mol. Biol. Cell, 8*:287-301, 1997). Staining of cells with these antibodies can be used to quantify the proportion of cells in these cell cycle phases by immuno-histochemistry or western blot analysis.

Another method for identification of cells in different phases of the cell cycle includes radioisotope labeling. For example, the ability of gemcitabine to arrest tumor cells in S phase has been assessed in multiple tumor types. Gandhi *et al* (*J. Clin. Ocol., 20*:665-73, 2002) disclosed a method for assessing S phase arrest in acute myelogenous leukemia patients after treatment with gemcitabine. Patients received gemcitabine at a constant dose of 10/mg/m^2/min for various durations of time and tumor cells isolated from blood of patients 24 hours after the start of therapy to determine the number of cells in S phase arrest. Cells were plated in triplicate (2 x 10^6) in RPMI-1640/10% Fetal bovine serum and 1 μCi of [^{3}H]thymidine. Cells were allowed to incubate for 30 minutes and thymidine incorporation measured. A decrease in radioisotope uptake after gemcitabine treatment indicates whether the cells are arrested in S phase, and the duration of the S phase arrest.

The first of the foregoing techniques was used to illustrate the influence of camptothecin, a well known chemotherapeutic agent that, in low doses, activates Chk1 at the S-phase, as shown in Table 2.

TABLE 2

HT29 Cells:	G1 (%)	S (%)	G2/M* (%)
In the absence of Chk1 activator (asynchronous)	34.2	45.7	14.5
After low-level camptothecin treatment	6.75	80.86	7

*Combined total in G2+M phase.

Two cell samples, each containing the same human carcinoma cell line (HT29) were prepared. Using propidium iodide to monitor DNA content, the percentage of cells the G1, S, and G2/M phases of the cell cycle were measured before and after contact with low levels of camptothecin. (Because priopidium iodide indicates total DNA content, this technique does not distinguish between cells in G2 vs. M phase. Accordingly, data reported in the G2/M column of Table 1 shows the total percentage of cells of the population in G2+M-phases.) The first sample was measured to establish the percentage of cells present in each phase asynchronous cell cycling, i.e., in the absence of Chk1 activator. Specifically, in the absence of Chk1 activator, 34.2% of the cells in the sample were in the G1 phase; 45.7% of the cells were in S phase; and 14.5% of the cells were in G2/M phase. The second sample was contacted with low levels of camptothecin (20 nM for 24 hours). Camptothecin is a Chk1 activator. At low levels, its target phase is S phase. As Table 1 shows, the camptochecin increased the percentage of cells in S phase from 45.7% to more than 80%, and decreased the percentage of cells in other phases. Thus, the Chk1 activator caused the percentage of cells in the cell population to synchronize to the degree shown in its target phase.

In the present invention, Chk1 activator is contacted with the cell population in an amount and for a time sufficient to substantially synchronize cell cycle arrest at the target phase for the Chk1 activator used, prior to contacting the population with Chk1 inhibitor. Ideally, the cell population undergoes optimal synchronization prior to contact with Chk1 inhibitor. For optimal synchronization, a maximum percentage of cells in the population to are allowed to "pile up" or arrest in the target phase for the activator used, with a minimum percentage having progressed

into mitosis. However, those skilled in the art will appreciate that lesser degrees of cell cycle synchronization prior to contact with the Chk1 inhibitor will provide some benefit. Thus, "substantial synchronization" includes any degree of synchronization of cell cycle arrest, including optimal, that results in a cytotoxic effect greater than that seen without use of Chk1 inhibitor, or greater than that seen with co-administration of Chk1 activator and inhibitor, or greater than that seen when the cells are contacted with Chk1 inhibitor prior to Chk1 activator. The degree of cell cycle arrest corresponding to or exceeding these references qualifies as "substantial synchronization" and is considered within the scope of this invention.

As non-limiting examples, treatment with a Chk1 inhibitor may follow at least about a 10% increase in the number of aberrantly proliferating cells in the target phase of the Chk1 activator used; optionally at least about 20%, optionally at least about 50%, optionally at least about a 100% increase; optionally at least about a 150% increase; optionally at least about a 200% increase; optionally at least about a 250% increase; optionally at least about a 300% increase; optionally at least about a 350% increase; optionally at least about a 400% increase, optionally at least about 450% increase, or optionally about 500% increase, as compared to the number of aberrantly proliferating cells present in such phase in the absence of a Chk1 activator. These ranges are merely exemplary, however, and dependent upon cell type, Chk1 activator used, and other factors readily discernable to those skilled in the art. Those skilled in the art will appreciate that other ranges and sub-ranges of percent increase are also contemplated as being within the scope of this invention without limitation. Those skilled in the art will also appreciate that the maximum percent increase for any particular cell population sample will be limited by various factors, including percentage of cells present in the target phase of the population prior to Chk1 activator contact.

As indicated above, upon achieving substantial synchronization of cell cycle arrest in the cell population, the present invention calls for contacting the cell population with a Chk1 inhibitor in an amount and for a time sufficient to substantially abrogate the cell cycle arrest. The term "substantially abrogate" is used to indicate that complete abrogation of all arrested cells may not be necessary for efficacy. Those skilled in the art will appreciate that a sufficient degree of cell cycle checkpoint abrogation may be achieved to disrupt cell cycle checkpoint mechanisms

and allow cells to pass to a subsequent phase in the cell cycle with unrepaired DNA damage sufficient to cause cell death or otherwise slow or stop aberrant cell proliferation.

Those skilled in the art will appreciate how to convert information concerning cell cycle synchronization and abrogation to practical use in the clinic or laboratory. For example, for any given cell line, Chk1 activator, and Chk1 inhibitor, the dose and time to achieve substantial cell cycle synchronization and substantial abrogation, respectively, may be measured *ex vivo*. *Ex vivo* measurements may then be applied to the clinic as a practical surrogate for direct measurement of the percentage of cells in various phases of the cell cycle.

In determining such measurements, those skilled in the art will appreciate that the duration of Chk1 activator contact with the cell population may, as indicated above, be influenced by the cell type exhibiting unwanted cell proliferation. Like most cells, aberrantly proliferating cells do not cycle at a universal rate. Some types proliferate faster than others, *i.e.*, have a faster doubling time. Thus, for example, treatment of a tumor cell type with a fast doubling time (e.g., pancreatic cancer or melanoma) may require shorter treatment with Chk1 activator to substantially synchronize cell cycle arrest, while treatment of a tumor with a slower doubling time (e.g., some colon, breast or prostate tumors) would require longer contact with Chk1 activator, all other things being equal, to induce substantially synchronous cell cycle arrest.

Times effective to allow substantial cell cycle synchronization by the Chk1 activator may vary from a few minutes up to 96 hours or more. In some embodiments, it may be preferable or desirable to administer Chk1 activator for up to several weeks or more, as determined by the attending physician or technician. Thus, Chk1 activator may contact the cell population for up to about 30 minutes, up to about 1 hour, up to about 2 hours, up to about 3 hours, up to about 4 hours, up to about 6 hours, up to about 12 hours, up to about 18 hours, up to about 24 hours, up to about 48 hours, up to about 72 hours or up to about 96 hours or more. Those skilled in the art will appreciate that the ranges of time expressed herein are merely exemplary; ranges and sub-ranges within those expressed are also within the scope of the invention.

Contact of the cell population with the Chk1 activator may occur in single doses or over a plurality of doses, according to methods well known in the art for the particular Chk1 activator or activators used. For example, the Chk1 activator may be given at a frequency of: 4 doses delivered as one dose per day at 4-day intervals (q4d x 4); 4 doses delivered as one dose per day at 3-day intervals (q3d x 4); 1 dose delivered per day at 5-day intervals (qd x 5); one dose per week for 3 weeks (qwk3); 5 daily doses, with two days rest, and another 5 daily doses (5/2/5); or, any dose regimen determined to be appropriate for circumstance. Some time may optionally be allowed to lapse between the last dose of Chk1 activator to achieve substantial synchronization of cell cycle arrest prior to contact with the first dose of Chk1 inhibitor as necessary. Similar regimens may be used when Chk1 activator is chemotherapeutic or radiotherapeutic. Additional radiotherapeutic doses are well known to those of ordinary skill in the art.

Contact of the cell population with the Chk1 inhibitor may likewise occur at any dose and time sufficient to achieve substantial abrogation of the cell cycle checkpoint. Typically, though not necessarily, such times include up to about 72 to about 96 hours, depending upon various factors such as those discussed above. In some embodiments, it may be desirable or necessary to administer Chk1 inhibitor over a period of up to about several weeks or more, as determined by the attending physician or technician. Thus, Chk1 inhibitor may typically be administered for up to about 1 hour, up to about 2 hours, up to about 3 hours, up to about 4 hours, up to about 6 hours, up to about 12 hours, up to about 18 hours, up to about 24 hours, up to about 48 hours, or up to about 72 hours. Those skilled in the art will appreciate that the ranges of time expressed herein are merely exemplary; ranges and sub-ranges within those expressed are also within the scope of the invention.

The Chk1 inhibitor may be administered over a plurality of doses. For example, the Chk1 inhibitor may be given at a frequency of: 4 doses delivered as one dose per day at 4-day intervals (q4d x 4); 4 doses delivered as one dose per day at 3-day intervals (q3d x 4); 1 dose delivered per day at 5-day intervals (qd x 5); one dose per week for 3 weeks (qwk3); 5 daily doses, with two days rest, and another 5 daily doses (5/2/5); or, any dose regimen pre-determined to be appropriate for the circumstance.

Use of the invention is indicated in treatment of any condition involving aberrant cell proliferation, including cancerous and non-cancerous cell proliferation. In one aspect, treatment may be of any condition responsive to agents that activate cell cycle arrest or are responsive to inhibitors of cell cycle checkpoint proteins.

Cancers include tumors or neoplasms derived from growths of tissue cells wherein multiplication of cells is uncontrolled and progressive. Some such neoplasms are benign, but others are termed "malignant," and can lead to death of the organism. Malignant neoplasms are distinguished from benign growths in that, in addition to exhibiting aggressive cellular proliferation, the malignant neoplasms can invade surrounding tissues and metastasize. Moreover, malignant neoplasms are characterized by showing a greater loss of differentiation (greater "dedifferentiation") and organization relative to one another and surrounding tissues. (This property is called "anaplasia.")

Cancers treatable by the present invention include solid tumors such as carcinomas and sarcomas. Carcinomas derive from epithelial cells which infiltrate (i.e., invade) surrounding tissues and give rise to metastases. Adenocarcinomas are carcinomas derived from glandular tissue, or from tissues that form recognizable glandular structures. Sarcomas are tumors whose cells are embedded in a fibrillar or homogeneous substance, like embryonic connective tissue. The invention also enables treatment of cancers of the myeloid or lymphoid systems, including leukemias, lymphomas, and other cancers that typically are not present as a tumor mass, but are distributed in the vascular or lymphoreticular systems.

Further contemplated are cancers including, but not limited to, myxoid and round cell carcinomas, human soft tissue sarcomas including Ewing's sarcoma, cancer metastases including lymphatic metastases, squamous cell carcinomas particularly of the head and neck, esophageal squamous cell carcinomas, oral carcinomas, blood cell malignancies, including multiple myelomas, leukemias, including acute lymphocytic leukemias, acute nonlymphocytic leukemias, chronic lymphocytic leukemias, chronic myelocytic leukemias, and hairy cell leukemias, effusion lymphomas (body cavity based lymphomas), thymic lymphoma lung cancers (including small cell carcinomas of the lungs, cutaneous T cell lymphomas, Hodgkin's lymphomas, non-Hodgkin's lymphomas, cancers of the adrenal cortex,

ACTH-producing tumors, non-small cell lung cancers, breast cancers, including small cell carcinomas and ductal carcinomas), gastro-intestinal cancers (including stomach cancers, colon cancers, colorectal cancers, and polyps associated with colorectal neoplasias), pancreatic cancers, liver cancers, urological cancers (including bladder cancers, such as primary superficial bladder tumors, invasive transitional cell carcinomas of the bladder, and muscle-invasive bladder cancers), prostate cancers, malignancies of the female genital tract (including ovarian carcinomas, primary peritoneal epithelial neoplasms, cervical carcinomas, uterine endometrial cancers, vaginal cancers, cancers of the vulva, uterine cancers and solid tumors in the ovarian follicle), malignancies of the male genital tract (including testicular cancers and penile cancers), kidney cancers (including renal cell carcinomas), brain cancers (including intrinsic brain tumors, neuroblastomas, astrocytic brain tumors, gliomas, and metastatic tumor cell invasions in the central nervous system), bone cancers (including osteomas and osteosarcomas), skin cancers (including malignant melanomas, tumor progressions of human skin keratinocytes, basal cell carcinomas, and squamous cell cancers), thyroid cancers, retinoblastomas, neuroblastomas, peritoneal effusions, malignant pleural effusions, mesotheliomas, Wilms's tumors, gall bladder cancers, trophoblastic neo-plasms, hemangiopericytomas, and Kaposi's sarcomas.

Use of the invention is also contemplated in treatment of conditions involving non-cancerous aberrantly proliferating cells. Such conditions include, but are not limited to, atherosclerosis, restenosis, vasculitis, nephritis, retinopathy, renal disease, proliferative skin disorders, psoriasis, keloid scarring, actinic keratosis, Stevens-Johnson Syndrome, rheumatoid arthritis (RA), systemic-onset juvenile chronic arthritis (JCA), osteoporosis, systemic lupus erythmatosis, hyperproliferative diseases of the eye including epithelial down growth; proliferative vitreoretinopathy (PVR); diabetic retropathy, Hemangio-proliferative diseases, ichthyosis, or papillomas.

Non-cancerous conditions treatable by the present invention may also include inflammation and inflammatory diseases, conditions, or disorders. Examples of such indications include, but are not limited to, rheumatoid arthritis, psoriasis, vitiligo, Wegener's granulomatosis, and systemic lupus erythematosus (SLE). Treatment of arthritis, Wegener's granulomatosis, and SLE often involves the use of

immunosuppressive therapies, such as ionizing radiation, methotrexate, and cyclophosphamide. Psoriasis and vitiligo commonly are treated with ultraviolet radiation (UV) in combination with psoralen. Such treatments typically induce, either directly or indirectly, DNA damage. Inhibition of Chk1 activity within the offending immune cells renders the cells more sensitive to control by these standard treatments. In general, Chk1 inhibitors useful in the invention may optionally be used to potentiate control of inflammatory disease cells when administered in combination with immunosuppressive drugs.

Animal models of some of the foregoing cancerous and non-cancerous conditions treatable by the present invention include for example: athymic nude mice injected with viable cancer cells from the HL60 cell line (human non-small cell lung cancer), athymic nude mice injected with Panc-01 human tumor cells (human pancreatic cancer), athymic nude mice injected with A375 human tumor cells (human melanoma), athymic nude mice injected with SKMES lung cancer cells (human lung cancer), athymic nude mice injected with SKOV-3.ip. ovarian carcinoma cells (human ovarian cancer), athymic nude mice injected with MDA-MB-361 breast cancer cells (human breast cancer), rats injected with 137-62 cells (breast cancer), and c56BL/Ka mice (cpdm/cpdm) (human psoriasis) (Gijbels et al., Exp. Dermatol., 9:351-358 (2000).

Chk1 inhibitors of the invention are contemplated for use in a composition comprising Chk1 inhibitors in a pharmaceutically acceptable diluent or carrier. In one aspect, the pharmaceutical composition comprises Chk1 inhibitors as set out above.

Formulations of the present invention can be administered in a standard manner for the treatment of the indicated diseases, such as orally, parenterally, transmucosally (e.g., sublingually or via buccal administration), topically, transdermally, rectally, via inhalation (e.g., nasal or deep lung inhalation). Parenteral administration includes, but is not limited to intravenous, intra-arterial, intraperitoneal, subcutaneous, intramuscular, intrathecal and intra-articular. Parenteral administration also can be accomplished using a high pressure technique, like POWDERJECT™.

For oral administration, including buccal administration, the composition can be in the form of tablets or lozenges formulated in conventional manner. For example, tablets and capsules for oral administration can contain conventional excipients such as binding agents (for example, syrup, acacia, gelatin, sorbitol, tragacanth, mucilage of starch, or polyvinylpyrrolidone), fillers (for example, lactose, sugar, microcrystalline cellulose, maize-starch, calcium phosphate, or sorbitol), lubricants (for example, magnesium stearate, stearic acid, talc, polyethylene glycol or silica), disintegrants (for example, potato starch or sodium starch glycolate), or wetting agents (for example, sodium lauryl sulfate). The tablets can be coated according to methods well known in the art.

Alternatively, the compounds of the present invention can be incorporated into oral liquid preparations such as aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, for example. Moreover, formulations containing these compounds can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can contain conventional additives, for example suspending agents, such as sorbitol syrup, methyl cellulose, glucose/sugar syrup, gelatin, hydroxyethylcellulose, hydroxypropylmethylcellulose, carboxymethylcellulose, aluminum stearate gel, and hydrogenated edible fats; emulsifying agents, such as lecithin, sorbitan monooleate, or acacia; nonaqueous vehicles (which can include edible oils), such as almond oil, fractionated coconut oil, oily esters, propylene glycol, and ethyl alcohol; and preservatives, such as methyl or propyl p-hydroxybenzoate and sorbic acid.

Such preparations also can be formulated as suppositories, e.g., containing conventional suppository bases, such as cocoa butter or other glycerides. Compositions for inhalation typically can be provided in the form of a solution, suspension, or emulsion that can be administered as a dry powder or in the form of an aerosol using a conventional propellant, such as dichlorodifluoromethane or trichlorofluoromethane. Typical topical and transdermal formulations comprise conventional aqueous or nonaqueous vehicles, such as eye drops, creams, ointments, lotions, and pastes, or are in the form of a medicated plaster, patch, or membrane.

Additionally, compositions of the present invention can be formulated for parenteral administration by injection or continuous infusion. Formulations for injection can be in the form of suspensions, solutions, or emulsions in oily or aqueous

vehicles, and can contain formulation agents, such as suspending, stabilizing, and/or dispersing agents. Alternatively, the active ingredient can be in powder form for constitution with a suitable vehicle (e.g., sterile, pyrogen-free water) before use.

A composition in accordance with the present invention also can be formulated as a depot preparation. Such long acting formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Accordingly, the compounds of the invention can be formulated with suitable polymeric or hydrophobic materials (e.g., an emulsion in an acceptable oil), ion exchange resins, or as sparingly soluble derivatives (e.g., a sparingly soluble salt).

The compounds useful according to the invention may be conjugated or linked to auxiliary moieties that promote any property of the compounds that may be beneficial in methods of therapeutic use. Such conjugates can enhance delivery of the compounds to a particular anatomical site or region of interest (e.g., a tumor), enable sustained therapeutic concentrations of the compounds in target cells, alter pharmacokinetic and pharmacodynamic properties of the compounds, and/or improve the therapeutic index or safety profile of the com-pounds. Suitable auxiliary moieties include, for example, amino acids, aligopeptides, or polypeptides, e.g., antibodies such as monoclonal anti-bodies and other engineered antibodies; and natural or synthetic ligands to receptors in target cells or tissues. Other suitable auxiliaries include fatty acid or lipid moieties, to promote biodistribution or uptake of the compound by target cells (see, e.g., Bradley et al., Clin. Cancer Res. (2001) 7:3229.

It is further contemplated that the method of the invention comprises administration of at least one agent to reduce side effects resulting from treatment of the subject. In one aspect, the side-effect reducing agent comprises at least one growth factor. In a related aspect, the side-effect reducing agent comprises at least one cytokine, at least one lymphokine, or at least one hematopoeitic factor. Growth factors, cytokines, and hematopoeitic factors useful in the methods of the invention include, but are not limited to, M-CSF, GM-CSF, TNF, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IFN, TNF, G-CSF, Meg-CSF, GM-CSF, thrombopoietin, stem cell factor, erythropoietin, angiopoietins, including Ang-1, Ang-2, Ang-4, Ang-Y, and/or the human angiopoietin-like polypeptide, vascular endothelial growth factor (VEGF), angiogenin, bone morphogenic protein-1 (BMP-1), BMP-2, BMP-3, BMP-4, BMP-5,

BMP-6, BMP-7, BMP-8, BMP-9, BMP-10, BMP-11, BMP-12, BMP-13, BMP-14, BMP-15, BMP receptor IA, BMP receptor IB, brain derived neurotrophic factor, ciliary neutrophic factor, ciliary neutrophic factor receptor cytokine-induced neutrophil chemotactic factor 1, cytokine-induced neutrophil chemotactic factor 2, cytokine-induced neutrophil chemotactic factor 2, endothelial cell growth factor, endothelin 1, epidermal growth factor, epithelial-derived neutrophil attractant, fibroblast growth factor (FGF) 4, FGF 5, FGF 6, FGF 7, FGF 8, FGF 8b, FGF 8c, FGF 9, FGF 10, FGF acidic, FGF basic, glial cell line-derived neutrophic factor receptor 1, glial cell line-derived neutrophic factor receptor 2, growth related protein, growth related protein, growth related protein, growth related protein, heparin binding epidermal growth factor, hepatocyte growth factor, hepatocyte growth factor receptor, insulin-like growth factor I, insulin-like growth factor receptor, insulin-like growth factor II, insulin-like growth factor binding protein, keratinocyte growth factor, leukemia inhibitory factor, leukemia inhibitory factor receptor, nerve growth factor nerve growth factor receptor, neurotrophin-3, neurotrophin-4, placenta growth factor, placenta growth factor 2, platelet-derived endothelial cell growth factor, platelet derived growth factor, platelet derived growth factor A chain, platelet derived growth factor AA, platelet derived growth factor AB, platelet derived growth factor B chain, platelet derived growth factor BB, platelet derived growth factor receptor, platelet derived growth factor receptor, pre-B cell growth stimulating factor, stem cell factor, stem cell factor receptor, transforming growth factor (TGF), TGF, TGF 1, TGF 1.2, TGF 2, TGF 3, TGF 5, latent TGF 1, TGF, binding protein I, TGF binding protein II, TGF binding protein III, tumor necrosis factor receptor type I, tumor necrosis factor receptor type II, urokinase-type plasminogen activator receptor, vascular endothelial growth factor, and chimeric proteins and biologically or immunologically active fragments thereof.

EXAMPLES

The following examples illustrate various non-limiting embodiments of the invention and/or provide support therefore. Example 1 compares the present invention to co-administration of Chk1 activator and Chk1 inhibitor in an art-recognized in vitro model. Example 2 provides a similar comparison using a mitotic index assay. Example 3 compares the present invention to co-administration of Chk1 activator and Chk1 inhibitor in an animal tumor model. Example 4 describes a

sensitive assay that may be used to measure Chk1 inhibitor activity in animal models. Example 5 demonstrates that selective Chk1 inhibitors are capable of abrogating DNA damage-induced G2 and S phase checkpoints. Example 6 demonstrates that Chk1 inhibitor is taken up by tumor cells in the presence of Chk1 activator in an art recognized xenograft tumor model. Example 7 describes the use of the previously exemplified assay to determine the effect of Chk1 inhibitors on cell cycle arrest. This assay is again used in Example 8 to provide an example of the determination of the optimal dose and time of Chk1 activator required to achieve selective cell cycle synchronization. Example 9 describes an assessment of the optimal contact time of a population of aberrantly proliferating cells with a Chk1 inhibitor to achieve substantial abrogation of cell cycle arrest. Example 10 describes an assessment of a dose response relationship between Chk1 inhibitor and abrogation of cell cycle arrest. Finally, Example 11 describes an assessment of optimal dose of Chk1 inhibitor for use in an embodiment of the invention.

EXAMPLE 1

Contacting Aberrantly Proliferating Cells With Chk1 Inhibitor After Substantial Cell Cycle Synchronization By Chk1 Activator Showed Better Anti-Proliferative Activity Than Co-Administration In A Non-Small Cell Lung Cancer Cancer Animal Model

A method of the invention provided an improved antiproliferative effect over co-administration in an art-recognized in vitro tumor model. In the experiment, gemcitabine was used as the Chk1 activator and a diaryl urea compound according to Keegan et al., PCT/US02/06452, was used as the selective Chk1 inhibitor. (The same Chk1 inhibitor was used in the examples that follow.) The target phase of gemcitabine is the S phase of the cell cycle. A non-small cell lung tumor xenograft tumor model, H460, was the art-recognized in vitro tumor model.

Nude mice were engrafted with H460 tumor cells and allowed to grow to an average of 75 mm3. Tumor-bearing mice were then treated with vehicle, gemcitabine or gemcitabine plus 400 mg/kg selective Chk1 inhibitor. The gemcitabine was administered at a dose of 160 mg/kg q3d x3 either simultaneously with the Chk1 inhibitor (co-administration) or, according to the invention, 18 hours prior to the Chk1 inhibitor to allow for S phase synchronization.

Tumors were measured every 2-3 days. On day 10, the median tumor volume for the vehicle group was 10 times the starting volume, while the gemcitabine alone group was four times the starting volume. The gemcitabine plus Chk1 inhibitor co-administration group was also four times the starting volume. The gemcitabine followed by Chk1 inhibitor group was only 1.1 times the starting volume. This experiment demonstrates that pretreatment with gemcitabine in an amount and for a time sufficient to substantially synchronize the tumor cells prior to checkpoint release by the Chk1 inhibitor leads to greater anti-tumor activity than co-administration of the two agents together.

EXAMPLE 2

Contacting Aberrantly Proliferating Cells With Chk1 Inhibitor After Substantial Cell Cycle Synchronization By Chk1 Activator Reduced Required Exposure Time To Chk1 Inhibitors In A Mitotic Index Assay

Chk1 inhibitors were tested in a cell-based proliferation assay for the ability to sensitize tumor cells to ionizing radiation or chemotherapy agents. Chk1 inhibitors were tested in combination with 5-FU, gemicitabine, ionizing radiation, camptothecin, etoposide, hydroxyurea, fludarabine, ara-C and aphidacolin. For each experiment, a serial dilution of each compound in combination with a ten-point dilution of each chemotherapy agent was included, in order to determine the concentration of chemotherapeutic required to inhibit the growth of 90% (GI90) of the cells in the presence and absence of the Chk1 inhibitor. This was called the fold sensitization. Fold sensitization was plotted as a function of Chk1 inhibitor concentration and the amount of drug required to yield two-fold sensitization was calculated. This concentration is referred to as the ECTFS or, the effective concentration of inhibitor required for yielding two-fold sensitization. Another parameter analyzed was the fold sensitization achieved at the LD50 (the dose of compound alone that inhibits growth of 50% of cells) for the compound. These two values allow direct ranking of both the potency and toxicity of molecules with respect to one another.

The sensitization assay described above was used to assess the ability of the Chk1 inhibitors to promote cell death after contact with a selective Chk1 inhibitor according to an embodiment of the invention. This in vitro assay is believed to correlate to anti-tumor activity of the Chk1 inhibitors in vivo. The sensitization

studies indicated that, in the samples tested, if gemcitabine and the Chk1 inhibitor were dosed simultaneously, the exposure time required for a Chk1 inhibitor to yield maximal sensitization (14 fold sensitization) was approximately 24 hours. However, if cells were treated first with gemcitabine for approximately 2 hours and the cells allowed approximately 24 hours to arrest in S phase before treating with the Chk1 inhibitor, as little as 4-6 hours of inhibitor exposure led to maximum sensitization (over 12-fold sensitization). In contrast, simultaneous treatment of gemcitabine and the Chk1 inhibitor for 6 hours resulted in no sensitization in the samples tested. These data suggest that allowing aberrantly proliferating cells to substantially synchronize cell cycle arrest before administering Chk1 inhibitor reduces the required time of exposure to Chk1 inhibitors to result in tumor cell death in combination with a Chk1 activating agent.

EXAMPLE 3

Contacting Aberrantly Proliferating Cells With Chk1 Inhibitor After Substantial Cell Cycle Synchronization By Chk1 Activator Showed Better Anti-Proliferative Activity Than Co-Administration In A Colon Cancer Animal Model

Nude mice were engrafted with HT29 colon carcinoma cells and tumors were grown to 200 mm3 for 10 days. The HT-29 tumor-bearing mice were treated with vehicle, 600 mg/kg Chk1 inhibitor (p.o.), 160 mg/kg gemcitabine (i.p.) or the co-administration of gemcitabine and Chk1 inhibitor. Alternatively, mice were pretreated according to the invention with gemcitabine for 24 hours, dosed with Chk1 inhibitor on day 2, and allowed to rest on day 3. The treatment regimen was repeated four times. This dosing strategy combined the MTD dosing for gemcitabine (160 mg/kg q3d x 4, i.e. 4 doses delivered as one dose per day at 3-day intervals) with a gemcitabine pretreatment strategy.

Tumors were measured every 2-3 days until they reached 1200 mgs and then the animals were sacrificed. Median tumor growth delay, survival benefit and tumor regressions were measured. The median time for tumors to grow from 200 mm3 to 800 mm3 was 14.5 days longer in the animals treated with gemcitabine then Chk1 inhibitor compared to animals treated with gemcitabine alone. The survival benefit was 15 days greater in mice treated with the combination therapy over gemcitabine alone.

In summary, substantial synchronization of the tumor cells in S-phase by gemcitabine followed by checkpoint release via the Chk1 inhibitor resulted in a significant improvement in the anti-tumor activity. Whereas co-administration resulted in a 4 day growth delay as described in Example 6, pretreatment with gemcitabine according to the invention resulted in a 14.5 day tumor growth delay.

EXAMPLE 4

A Sensitive Assay to Measure Chk1 Inhibitor Activity in Animal Models

The following sensitive assay was developed to measure Chk1 inhibitor activity in rodent tumor models. In particular, the assay may be used, inter alia, to measure the ability of Chk1 inhibitors to block Chk1 function in the tumor model, and to allow for assessment of conditions that facilitate Chk1 inhibitors' access to the molecular target.

The ability of selective Chk1 inhibitors to abrogate a chemotherapy-induced checkpoint was measured using a quantitative immunofluourescent assay that measures mitotic index by monitoring histone H3 phosphorylation on serine 10 (H3-P), a mitosis-specific event (Ajiro et al., J Biol Chem. 271:13197-201. 1996; Goto et al., J Biol Chem.;274:25543-9, 1999). The assay protocol was as follows. Tumors from rodents treated or untreated with Chk1 activator (in the present study, chemotherapy agent) and/or Chk1 inhibitor, were excised and paraffin embedded. The tumors are cut into 6 micron thick slices and mounted on glass slides. The paraffin was removed from the slides by 3 minute successive treatments with xylene, 100% ethanol, 95% ethanol, 70% ethanol and deionized water. The slides are then heated to 95°C in 10mM sodium citrate for 10 minutes followed by a 20 minute cooling step. The slides are blocked for 30 minutes with Block buffer (20% normal human serum and 2% bovine serum albumin in phosphate buffered saline containing 0.05% Triton X-100 (PBST)). The anti-phospho histone H3 antibody (Upstate Biotech, Cat. #06-570) is diluted 1:200 in the Block buffer and incubated with the slides for one hour. The slides are washed 3 times 5 minutes in PBST. The secondary antibody, donkey anti-rabbit rhodamine (Jackson, cat #711-295-152) was added for 30 minutes. The slides were then washed twice in PBST and 75□M of 0.1□M/ml DAPI (Sigma) in PBS is added and allowed to stain for 30 minutes. The slides were then washed two more times in PBST and mounted with Vectashield

(Vector, cat # H-1400). Slides were viewed using fluorescence microscopy. The percentage of cells stained with H3-P antibody relative to total (DAPI stained) cells were quantified using Metamorph software (Universal Imaging Corporation, Version 4.6).

EXAMPLE 5

Selective Chk1 Inhibitors Abrogate DNA Damage-Induced G2 and S Phase Checkpoints

Previous studies have demonstrated that selective Chk1 inhibitors substantially abrogate the DNA damage-induced G2/M and S phase checkpoints. In the former, DNA damage was induced by ionizing radiation (IR), whose target phase is the G2 phase. In the latter, DNA damage was induced by chemotherapeutic agents whose target phase is the S phase. See published U.S. patent application 2003/0069284 and references cited therein.

Briefly, the Chk1 inhibitor abrogation of IR-induced G2 DNA damage checkpoint was assayed by mitotic index experiments. Approximately 1x106 HeLa cells were irradiated with 800 rads and incubated for 7 hours at 37° C. Because these cells are functionally p53 negative, they arrest exclusively in G2. Nocodazole was then added to a concentration of 0.5 μg/mL and incubated for 15 hours at 37° C. (The addition of nocodazole was designed to trap any cells that progressed through the G2 arrest in mitosis thus preventing them from further progressing into G1 and allowing for quantification of M phase cells.) A selective Chk1 inhibitor was added for 8 hours, and the cells harvested by centrifugation, washed once with PBS, then resuspended in 2.5 mL 75 mM KCl and centrifuged again. The cells then were fixed in 3 mL of freshly prepared cold, acetic acid: methanol (1:3) and incubated on ice for 20 minutes. Cells were pelleted, the fix solution was aspirated and the cells were resuspended in 0.5 mL of PBS. Mitotic spreads were prepared by pipeting 100 μL of the fixed cells onto a glass microscope slide and flooding the sample with 1 ml of fix solution. Slides were then air dried, stained with Wrights stain (Sigma, St. Louis, MO) for 1 minute, followed by one wash in water and one wash in 50% methanol. The presence of condensed chromosomes and lack of nuclear envelope identified mitotic cells. The selective Chk1 inhibitors (diarylurea compounds according to US 2003/0069284) tested resulted in an increase in the number of mitotic cells in the presence of irradiation, thereby demonstrating abrogation of the IR-induced G2 arrest.

This checkpoint abrogation results in an enhancement in the activity of CyclinB/cdc2, which is required for progression of cells into mitosis. Cells treated with IR followed by Chk1 inhibitor thus progress into mitosis with damaged DNA. These experiments confirm the hypothesis that Chk1 is involved in the IR-induced G2 arrest and that selective inhibitors of Chk1 allow cells to continue cycling in the presence of DNA damage.

Previous studies also demonstrated that selective Chk1 inhibitors abrogate the S phase checkpoint induced by CPT, Ara-C, gemcitibine, fludarabine and aphidicolin in HT29 cells. The S phase abrogation was induced by these agents in a dose-dependent manner and resulted in entry into mitosis despite DNA damage, resulting in cell death. (Microscopic analysis of mitotic cells treated with Chk1 inhibitor suggested that the chromosomes were improperly aligned on the mitotic spindles. Without wishing to be bound by theory, one hypothesis suggests that premature entry into mitosis results in defects in attachment of microtubules to kinetocores, inducing a spindle checkpoint and metaphase arrest, ultimately leading to death caused by mitotic catastrophe.)

In summary, these studies demonstrated that selective Chk1 inhibitors block Chk1 function at both G2/M and S phase, causing cells treated with DNA damaging agents to progress beyond the checkpoint with unrepaired DNA damage.

EXAMPLE 6

Chk1 Inhibitor Is Taken Up by Tumor Cells in the Presence of Chk1 Activator in a Xenograft Tumor Model.

In a xenograft tumor model, nude mice were engrafted with HT29 colon carcinoma tumors on the flank and allowed to grow to 200 mm3. Mice were then treated with either vehicle, 300 mg/kg Chk1 inhibitor, 20 mg/kg gemcitabine or co-administered with 300 mg/kg Chk1 inhibitor and 20 mg/kg gemcitabine two times, three days apart on Days 1 and 4. Treatment of tumor-bearing mice by co-administration of Chk1 inhibitor and gemcitabine resulted in a four day growth delay in tumors compared to gemcitabine alone.

To assess the diffusion of Chk1 inhibitors into tumor tissue, plasma and tissue levels of Chk1 inhibitor were measured. Using an Alzet pump, 500 mg/kg Chk1 inhibitor was administered to HT29 tumor-bearing mice in a continuous

delivery system over a 24 hour period. Plasma samples were taken and then tumors, kidney, liver, spleen and lung were harvested. Time points were collected at 1, 2, 4, 8 and 24 hours. Tissues were extracted and levels of Chk1 inhibitor were quantified. This experiment demonstrated that the Chk1 inhibitor showed penetration into normal and tumor tissue and reached a level of approximately 15 μM in tumor tissue and peaked in spleen tissue at 8 hours at approximately 20 μM. Thus, Chk1 inhibitors were readily taken up by the proliferating cells and deemed useful, in conjunction with Chk1 activating chemotherapeutic agents, as therapies for the treatment of proliferative diseases.

EXAMPLE 7

Use of H3-P Assay to Determine the Effect of Chk1 Inhibitors on Cell Cycle Arrest

The effect of selective Chk1 inhibitors on Chk1 activator induced cell cycle arrest may be assessed using the assay described above. In this example, gemcitabine was used in mice bearing HT29 tumors.

Mice bearing HT29 tumors were treated with vehicle, 100 mg/kg gemcitabine for 48 hours, or 100 mg/kg gemcitabine for 48 hours followed by the addition of Chk1 inhibitor for 24 hours. Tumors were removed, embedded in paraffin and HT29 tumor slices were stained with antibody against H3-P. Mice pretreated with gemicitabine for 48 hours followed by a 24-hour Chk1 inhibitor treatment demonstrated abrogation of the S phase checkpoint, showing approximately 14% mitotic cells compared to approximately 4% in gemcitabine treated mice. This experiment demonstrated that the Chk1 inhibitor allows S phase arrested tumor cells to progress out of the gemcitabine-induced cell cycle arrest and into mitosis.

Using this assay, the scheduling and timing of gemcitabine and Chk1 inhibitors may be optimized. The assay also allows, inter alia, for the measurement of biologically efficacious doses of Chk1 inhibitors and optimization of the Chk1 activator dose and/or pretreatment time.

EXAMPLE 8

Use of H3-P Assay to Determine Optimal Dose and Time to Achieve Cell Cycle Synchronization by Chk1 Activator

In a non-limiting embodiment, the H3-P assay discussed above may be used to determine an optimal degree of cell cycle arrest by Chk1 activator. In the present example, the Chk1 activator was gemcitabine, whose target phase is S phase. The animal model was HT29 tumor-bearing mice.

HT29 tumor-bearing mice were treated with 100 mg/kg gemcitabine intraperitoneally (i.p.) and mice were harvested at 1hr, 2hr, 4hr, 6hr, 12hr, 24hr, 48hr and 72 hr. Tumors from these animals were resected, paraffin embedded and stained with an antibody to H3-P followed by a counter-stained with DAPI. The percentage of mitotic cells (positive to H3-P) was quantified at each time point. The data indicated that most cells arrested in S phase between 12 and 24 hr after gemcitabine administration, with a mitotic index of approximately 1.5, compared to an index of approximately 3 at the 1-6 hr time points.

To confirm that low H3-P staining corresponds to S phase arrest, tumors were also stained with an S phase marker, phosphorylated Rb-Pser795. Tumor slices taken in the experiment above were stained with the Rb-Pser795 antibody (Cell Signaling Cat# 9301S) and the number of positive staining cells quantified. The results demonstrated that there were more Rb-P staining cells at 24, 48 and 72 hours than at earlier timepoints. Taken together, these data indicate that the optimal S phase arrest induced by gemcitabine in HT29 tumors occurred in the particular sample tested at 24-48 hours post-gemcitabine treatment.

The kinetics of S phase arrest in response to gemcitabine varies in tumors depending on their doubling time. The human non-small cell lung carcinoma, H460, and the rat breast cancer 137-62 tumors, which have faster doubling times than HT29 tumors (4.5 and 2 days respectively, compared to 10 days or HT29) show reduced H3-P staining at earlier times than HT29 tumors. In an experiment similar to that described above for HT29 cells, H460 and 137-62 were treated with gemcitabine and tumors were harvested at various timepoints. In both tumor types, the lowest H3-P staining is at 12 hours (compared to 48 hr in HT29 cells) and the cells exited S phase arrest at 24 hours in 137-62 cells and 48 hours in H460 cells.

These results suggest that faster growing tumors cycle around into S phase and arrest more rapidly than slower growing tumors. Furthermore, the faster the doubling time of the tumor, the faster they enter back into the cell cycle after gemcitabine arrest. Thus, the optimal gemcitabine pretreatment time may vary depending on the doubling time of the tumor. The fairly broad range of observed pretreatment times that resulted in an S phase arrest suggests that it will be practical to translate this regime to the clinic or laboratory.

EXAMPLE 9

An Assessment Of Optimal Contact Time With Chk1 Inhibitor Following Substantial Cell Cycle Synchronization

This example illustrates an assessment of the effects of Chk1 inhibitors on kinetics of the abrogation of the cell cycle arrest following substantial synchronization by Chk1 activator. In the present non-limiting example, a cell population comprising human colon carcinoma cell line HT29 was treated with 20 μM gemcitabine for two hours, the gemcitabine washed out, and cells allowed to substantially synchronize at S phase. After 18 hours, the cells were then treated with Chk1 inhibitor and time points taken from 30 minutes to 24 hours. Results showed that progression through the S phase checkpoint started at 2 hours and peaked at 8 hours, with approximately 80% of cells in mitosis. Levels of cells entering into mitosis dropped off by 24 hours, presumably because the cells began to die. These data suggest that the optimal time of exposure of HT29 cells to Chk1 inhibitor after gemcitabine-induced S phase arrest in the samples tested was 6-8 hours. It was observed that some cell lines that are sensitized to Chk1 inhibitors and gemcitabine (such as the 137-62 breast cell carcinoma) enter into mitosis after S phase arrest with this chemotherapy treatment. However, based on the cell sensitization data gathered, it is believed likely that in these cells the Chk1 inhibitors allow abrogation of the cell cycle checkpoint, but rather than progress into mitosis, they progress out of S phase and then die via apoptosis.

EXAMPLE 10

An Assessment Of Dose Response Of Chk1 Inhibitor Abrogation Following Substantial Cell Cycle Synchronization

To determine whether checkpoint abrogation by selective Chk1 inhibitor was dose dependent, HT29 tumor bearing mice were pretreated with gemcitabine and 32 hours later dosed with increasing doses of selective Chk1 inhibitor. After 18 hours, tumors were harvested and stained for H3-P as described above. Results indicated that entry into mitosis after checkpoint abrogation is dose dependent, with about 5% of cells in mitosis at 100 mg/kg of Chk1 inhibitor, increasing to approximately 11% at 400 mg/kg. The response is saturated at 400 mg/kg. These data confirm a dose-dependent response to Chk1 inhibitor up to a saturation point.

EXAMPLE 11

Dose Response of Tumors Treated With Chk1 Inhibitors and Gemcitabine

To determine an efficacious dose of Chk1 inhibitor following gemcitabine treatment and whether the dose-dependent checkpoint abrogation correlated with anti-tumor activity, a dose response experiment was performed.

Nude mice were engrafted with HT29 tumor cells and tumors allowed to develop for 10 days. The tumors at the start were approximately 100 mm3. Animals were treated with gemcitabine at the MTD (160 mg/kg) followed by Chk1 inhibitor at 50 mg/kg, 200 mg/kg or 400 mg/kg administered as in Example 1. Gemcitabine pretreatment time was 32 hours in this experiment, as the cell-based assay indicated this timepoint was optimal for this type of tumor. Analysis of tumor volume in each treatment regimen indicated that treatment of HT29 tumor bearing mice with the described therapy slowed tumor growth greater than gemcitabine alone, with either 200 mg/kg or 400 mg/kg Chk1 inhibitor plus gemcitabine again showing dose-dependent effects of the Chk1 inhibitor.

The present invention is not to be limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention, and compositions and methods which are functionally equivalent are within the scope of the invention. Indeed, numerous modifications and variations in the practice of the

invention are expected to occur to those skilled in the art upon consideration of the present preferred embodiments. Consequently, the only limitations that should be placed upon the scope of the invention are those that appear in the appended claims. All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.

We claim:

1. A method for inhibiting aberrant cell proliferation comprising contacting a cell population comprising aberrantly proliferating cells with a Chk1 activator to substantially synchronize cell cycle arrest among said aberrantly proliferating cells, and subsequently contacting said cell population with a selective Chk1 inhibitor to substantially abrogate said cell cycle arrest.

2. A method of claim 1, wherein said cell population is contacted with a Chk1 activator for from about 30 minutes to about 96 hours, and subsequently contacted with a selective Chk1 inhibitor for from up to about 1 hour to up to about 72 hours.

3. A method of claim 2, wherein said cell population is contacted with a Chk1 activator for from about 30 minutes to about 48 hours.

4. The method of claim 1, wherein said Ck1 activator induces substantial synchronization of cell cycle arrest at the target phase G1.

5. The method of claim 1, wherein said Chk1 activator induces substantial synchronization of cell cycle arrest at the target phase S.

6. The method of claim 1, wherein said Chk1 activator induces substantial synchronization of cell cycle arrest at the target phase G2.

7. The method of claim 1, wherein said cell population is *ex vivo*.

8. The method of claim 1, wherein said cell population is *in vivo*.

9. The method of claim 8, wherein said cell population is in a human.

10. The method of claim 1, wherein said Chk1 activator comprises at least one chemotherapeutic agent.

11. The method of claim 10, wherein said chemotherapeutic agent comprises an anti-metabolite, a DNA damaging agent, a cytokine, a covalent DNA-binding drug, a topoisomerase inhibitor, an anti-tumor antibiotic, a differentiation agent, a platinum complex, or an alkylating agent.

12. The method of claim 10, wherein said chemotherapeutic agent comprises gemcitabine.

13. The method of claim 1, wherein said Chk1 activator comprises ionizing or ultraviolet radiation.

14. The method of claim 13, wherein said ionizing radiation is administered in conjunction with a radiosensitizer and/or a photosensitizer.

15. A method of claim 1, wherein said cell population is contacted with a Chk1 inhibitor after a time sufficient to allow said Chk1 activator to induce a maximum degree of synchronization in said cell population of cell cycle arrest and a minimum number of cells in mitosis.

16. A method of claim 1, wherein the substantially synchronized cell cycle arrest achieved by contacting said cell population with said Chk1 activator comprises at least about a 50% increase in the number of aberrantly proliferating cells in the target phase of said Chk1 activator in comparison to the number of aberrantly proliferating cells in the target phase prior to contact with said Chk1 activator.

17. A method of claim 16, wherein said increase is at least about 100%.

18. A method of claim 17, wherein said increase is at least about 200%.

19. A method of claim 18, wherein said increase is at least about 300%.

20. A method of claim 19, wherein said increase is at least about 400%.

21. A method of claim 1, wherein said cell population is contacted with said Chk1 activator for at least one doubling period typical of aberrantly proliferating cells in said cell population.

22. A method of claim 1, wherein said cell population is contacted with said Chk1 activator for at least two doubling periods typical of aberrantly proliferating cells in said cell population.

23. The method of claim 1, further comprising determining the presence or absence of substantial synchronization of cell cycle arrest in a biological sample.

24. The method of claim 23 wherein the biological sample is a fluid sample or a tissue sample.

25. The method of claim 1, wherein said Chk1 inhibitor is administered over a plurality of doses.

26. The method of claim 25, wherein said doses comprise a frequency of (q4d x 4), (q3d x 4), (qd x 5), (qwk3), or (5/2/5).

27. The method of claim 1, wherein said aberrantly proliferating cells are cancerous.

28. The method of claim 27, wherein said cancerous cells comprise cells from myxoid and round cell carcinomas, locally advanced tumors, metastatic cancer, Ewing's sarcoma, cancer metastases, lymphatic metastases, squamous cell carcinomas, esophageal squamous cell carcinomas, oral carcinomas, multiple myelomas, acute lymphocytic leukemias, acute non-lymphocytic leukemias, chronic lymphocytic leukemias, chronic myelocytic leukemias, hairy cell leukemias, effusion lymphomas (body cavity based lymphomas), thymic lymphoma lung cancers, small cell carcinomas of the lung, cutaneous T cell lymphomas, Hodgkin's lymphomas, non-Hodgkin's lymphomas, cancers of the adrenal cortex, ACTH-producing tumors, non-small cell lung cancers, breast cancers, small cell carcinomas, ductal carcinomas, stomach cancers, colon cancers, colorectal cancers, polyps associated with colorectal neoplasias, pancreatic cancers, liver cancers, bladder cancers, primary superficial bladder tumors, invasive transitional cell carcinomas of the bladder, muscle-invasive bladder cancers, prostate cancers, ovarian carcinomas, primary peritoneal epithelial neoplasms, cervical carcinomas, uterine endometrial cancers, vaginal cancers, cancers of the vulva, uterine cancers and solid tumors in the ovarian follicle, testicular cancers, penile cancers, renal cell carcinomas, intrinsic brain tumors, neuroblastomas, astrocytic brain tumors, gliomas, metastatic tumor cell invasions in the central nervous system, osteomas and osteosarcomas, malignant melanomas, tumor progressions of human skin keratinocytes, squamous cell cancers, thyroid cancers, retinoblastomas, neuroblastomas, peritoneal effusions, malignant pleural effusions, mesotheliomas, Wilms's tumors, gall bladder cancers, trophoblastic neoplasms, hemangiopericytomas, Kaposi's sarcomas or other cancers treatable with chemotherapy agents or inhibitors of cell cycle checkpoint proteins.

29. The method of claim 1, wherein said aberrantly proliferating cells are non-cancerous.

30. The method of claim 27, wherein said non-cancerous cells comprise cells originating from atherosclerosis, restenosis, vasculitis, nephritis, retinopathy, renal disease, proliferative skin disorders, psoriasis, keloid scarring, actinic keratosis, Stevens-Johnson Syndrome, rheumatoid arthritis, systemic-onset juvenile chronic arthritis, osteoporosis, systemic lupus erythmatosis, hyperproliferative diseases of the eye including epithelial down growth, proliferative vitreoretinopathy (PVR), Hemangio-proliferative diseases, ichthyosis, or papillomas.

31. Use of a composition comprising at least one Chk1 inhibitor in the manufacture of a medicament for the inhibition of aberrant cell proliferation.

32. An article of manufacture comprising a Chk1 inhibitor and a label indicating a method according to claim 1.

ABSTRACT

The present invention relates to improved methods for inhibiting aberrant cell proliferation involving the scheduling of administration of Chk1 activators (e.g., chemotherapeutic agents) and Chk1 inhibitors. At least one Chk1 activator is administered at a dose and for a time sufficient to induce substantial synchronization of cell cycle arrest in proliferating cells. Upon achieving substantial phase synchronization, at lease one Chk1 inhibitor is administered to abrogate the cell cycle arrest and induce therapeutic cell death. The invention is useful with any Chk1 activator and any Chk1 inhibitor, and finds application in treating or preventing cancerous and non-cancerous aberrant cell proliferation.